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22 November 1996

COMPANY SANITIZED

Document Control Office (7404)
Office of Toxic Substances
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, DC 20460

ATTN: TSCA Section 8(e) Coordinator

[SANITIZED]

RE: TSCA Section 8(e) Notification of Furfural
[ ]

Gentlemen:

This TSCA Section 8(e) substantial risk notification concerning an In Vitro mammalian cytogenetic test with an independent repeat assay with furfural is being submitted by [ ]. The CAS Registry Number for the chemical is 98-01-1. The test was performed at Microbiological Associates, Inc., Rockville, Maryland, under their study No. [ ]. The information summarized below was received on November 21, 1996 via the final report (copy enclosed).

Initial assay and an independent repeat assay were performed with the chromosome aberration assay using Chinese hamster ovary (CHO) cells. The assays were done both in the presence and, absence of an Aroclor-induced S9-metabolic activation system. Dimethylsulfoxide was selected as the solvent of choice.

Results of the initial chromosome aberration assay using CHO cells exhibited statistically significant increases in chromosome aberrations relative to the solvent control in both the non-activated test system and the S9-activated test system. In the independent repeat assay, statistically significant increases in structural chromosome aberrations were observed in both the 20- and 44-hour exposure groups for both non-activated and S9-activated test systems. Also, the percentage of cells with numerical aberrations was statistically significantly increased above that of the solvent control in the 44-hour exposure group for both non-activated and S9-activated test systems.

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FINAL REPORT

Study Title

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IN VITRO MAMMALIAN CYTOGENETIC TEST WITH AN INDEPENDENT REPEAT ASSAY

Test Article

Furfural

Authors

Ramadevi Gudi, Ph.D.
Elizabeth H. Schadly, B.S.

Study Completion Date

November 19, 1996

Performing Laboratory

Microbiological Associates, Inc.
9630 Medical Center Drive
Rockville, Maryland 20850

Laboratory Study Number

[ ]

Sponsor

[ ]

STATEMENT OF COMPLIANCE

Study[ ]was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Ramadevi Gudi

Ramadevi Gudi, Ph.D.
Study Director

11/19/96
Date

QUALITY ASSURANCE STATEMENT

Study Title: *IN VITRO* MAMMALIAN CYTOGENETIC TEST WITH AN INDEPENDENT REPEAT ASSAY

Study Number: [ ]

Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 09 MAY 96, TO STUDY DIR 09 MAY 96, TO MGMT 09 MAY 96
PHASE: Protocol Review

INSPECT ON 21 JUN 96, TO STUDY DIR 26 JUN 96, TO MGMT 26 JUN 96
PHASE: Colcemid treatment of the test system

INSPECT ON 20 SEP 96-24 SEP 96, TO STUDY DIR 24 SEP 96, TO MGMT 27 SEP 96
PHASE: Draft Report

INSPECT ON 19 NOV 96, TO STUDY DIR 19 NOV 96, TO MGMT 19 NOV 96
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Diane B. Madsen
QUALITY ASSURANCE

11-19-96
DATE

IN VITRO MAMMALIAN CYTOGENETIC TEST WITH AN INDEPENDENT REPEAT ASSAY

FINAL REPORT

Sponsor: [ ]

Authorized Representative: [ ]

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9630 Medical Center Drive
Rockville, Maryland 20850

Test Article I.D.: **Furfural**

MA Study No.: [ ]

Test Article Description: **clear liquid**

Storage Conditions: **room temperature, protected from exposure to light**

Test Article Receipt: **May 2, 1996**

Study Initiation: **May 6, 1996**

Laboratory Supervisor: **Elizabeth H. Schadly, B.S.**

Study Director: Ramadevi Gudi 11/19/96
Ramadevi Gudi, Ph.D. Date

TABLE OF CONTENTS

SUMMARY

The test article, Furfural, was tested in the chromosome aberration assay using Chinese hamster ovary cells. The assay was performed in two phases. The first phase, the initial chromosome aberration assay was conducted to determine the dose range and to evaluate the clastogenic potential of the test article. The second phase, the independent repeat chromosome aberration assay, was performed to confirm the test system response to the test article seen in the initial assay. Both phases were conducted in the absence and presence of metabolic activation.

Dimethylsulfoxide (DMSO) was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at 500 mg/ml, the maximum concentration tested.

In the initial assay, the maximum dose tested was 5000 µg/ml. This dose was achieved using a concentration of 500 mg/ml, and a 50 µl dosing aliquot. The test article was soluble in treatment medium at all concentrations tested. In both the non-activated and S9-activated portions of the initial chromosome aberration assay, CHO cells were exposed to the test article for 6 hours. Metaphase cells were collected for microscopic evaluation at 20 hours after the initiation of treatment. Dose levels of 50, 150, 500, and 1500 µg/ml were selected for microscopic analysis in both the non-activated and S9-activated studies. Dose level 5000 µg/ml in the non-activated study was not evaluated due to excessive toxicity, and dose level 5000 µg/ml in the S9-activated study was not evaluated due to a lack of scorable cells. Substantial toxicity (cell growth inhibition) was observed at the highest dose level evaluated for chromosome aberrations, 1500 µg/ml, in the non-activated study. No substantial toxicity was observed at the highest dose level evaluated for chromosome aberrations, 1500 µg/ml, in the S9-activated study.

Statistically significant increases in chromosome aberrations relative to the solvent control group were observed in the non-activated test system at dose levels 150, 500, and 1500 µg/ml ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$). Statistically significant increases in chromosome aberrations relative to the solvent control group were observed in the S9-activated test system at dose levels 500 and 1500 µg/ml ($p \leq 0.05$ and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$).

Based on the results of the initial assay, an independent repeat chromosome aberration assay was conducted in the absence and in the presence of an Aroclor-induced S-9 metabolic activation system at dose levels of 94, 188, 375, 750, 1500, and 3000 µg/ml. The test article was soluble in treatment medium at all dose levels tested. In the non-activated portion of the independent repeat assay, CHO cells were exposed to the test article for 20 and 44 hours continuously; in the S9-activated portion of the assay the cells were exposed to the test article for 6 hours. Metaphase cells were collected for microscopic evaluation in both the non-activated and S-9 activated studies at 20 and 44 hours after the initiation of treatment. In the non-activated 20 hour exposure group dose levels of 94, 188, 375, and 750 µg/ml were selected for microscopic analysis, dose level

1500 µg/ml was not evaluated due to a lack of scorable cells and dose level 3000 µg/ml was not evaluated due to extreme toxicity. In the non-activated 44 hour exposure group dose levels 94, 188, and 375 µg/ml were selected for microscopic analysis, dose level 750 µg/ml was not evaluated due to a lack of scorable cells and dose levels 1500 and 3000 µg/ml were not evaluated due to extreme toxicity. Therefore only three test article dose levels were scored in the non-activated 44 exposure group. This constitutes a deviation from the protocol. This deviation was documented in the raw data with a deviation report and was concluded by the Study Director to have no significant effect on the integrity of the study. In the S9-activated 20 and 44 hour harvest groups, dose levels 94, 188, 375, and 750 µg/ml were selected for microscopic analysis. In the S9-activated 20 hour harvest dose levels 1500 and 3000 µg/ml were not evaluated due to lack of scorable cells and in the S9-activated 44 hour harvest groups, dose level 1500 µg/ml was not evaluated due to lack of scorable cells and 3000 µg/ml was not evaluated due to extreme toxicity. Toxicity (cell growth inhibition) was approximately 54% and 81% at the highest dose levels evaluated for chromosome aberrations in the non-activated studies, 750 µg/ml and 375 µg/ml, at the 20 and 44 hour harvests, respectively. Toxicity (cell growth inhibition) was approximately 44% and 81% at the highest dose level evaluated for chromosome aberrations in the S9-activated studies, 750 µg/ml, at the 20 and 44 hour harvests. respectively.

Statistically significant increases in structural chromosome aberrations were observed in the non-activated 20 hour exposure group at dose levels 375 and 750 µg/ml and at dose levels 188 and 375 µg/ml in the non-activated 44 hour exposure group ($p \leq 0.01$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$) in both the 20 and 44 hour exposure groups. The percentage of cells with numerical aberrations was significantly increased above that of the solvent control at dose levels 94 and 188 µg/ml in the non-activated 44 hour exposure group ($p \leq 0.05$ and $p \leq 0.01$, respectively, Fisher's exact test). Statistically significant increases in structural chromosome aberrations were observed in the S9-activated 20 hour harvest group at dose levels 94, 188, 375, and 750 µg/ml ($p \leq 0.01$, Fisher's exact test) and in the S9-activated 44 hour harvest group at dose levels 188, 375, and 750 µg/ml ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$) in both the S9-activated 20 and 44 hour harvest groups. The percentage of cells with numerical aberrations was significantly increased above that of the solvent control at dose levels 94, 375 and 750 µg/ml ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.01$, respectively, Fisher's exact test) in the S9 activated 44 hour harvest group. The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$).

All criteria for a valid study were met as described in the protocol. Under the conditions of this study, test article Furfural was concluded to be positive for the induction of structural and numerical chromosome aberrations in the *in vitro* mammalian cytogenetics test.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Furfural, was received by Microbiological Associates, Inc. on May 2, 1996 and was assigned the code number[ ] The test article was characterized by the Sponsor as a clear, yellowish to dark amber, mobile liquid that should be stored in a cool, dry, well ventilated location with an expiration date of November 1, 1996. Upon receipt, the test article was described as a clear liquid and was stored at room temperature, protected from exposure to light. The solvent used to deliver Furfural to the test system was dimethylsulfoxide (DMSO), obtained from the Fisher Chemical Company.

Mitomycin C (MMC, CAS 50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 8 and 15 µg/ml for use as the positive control in the non-activated test system. Cyclophosphamide (CP, CAS 6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 0.5, 1 and 2 mg/ml for use as the positive control in the S9-activated test system. For each positive control only one dose level exhibiting a sufficient number of scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S9 reaction mixture was used in the untreated control.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. In order to assure the karyotypic stability of the cell line, cells were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981).

Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for its

ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin and 100 µg streptomycin/ml, and 2 mM L-glutamine).

Initial and Independent Repeat Assays

The chromosome aberration assays were performed using standard procedures (Evans 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive, solvent, and untreated controls. CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask for cell collection times of 20 hours and at approximately 3 x 10^5 cells/25 cm^2 flask for cell collection times in excess of 20 hours. All cultures were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours prior to test article treatment. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 µg streptomycin/ml, and 2 mM L-glutamine) for the non-activated study or 4 ml serum-free medium and 1 ml S-9 reaction mixture for the activated study, to which was added 50 µl of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S9 reaction mixture was also included. In the initial chromosome aberration assay the cells were exposed to the test article for 6 hours in both the non-activated and S9-activated test systems. The cells were harvested at a single time point of 20 hours (1.5 times the cell cycle) after the initiation of treatment. In the independent repeat chromosome aberration assay the cells were exposed to the test article for either 20 or 44 hours in the non-activated test system and harvested at either 20 hours or 44 hours (1.5 times the cell cycle plus 24 hours) after the initiation of treatment. In the S9-activated test system the cells were exposed for 6 hours and harvested at either 20 hours or 44 hours (1.5 times the cell cycle plus 24 hours) after the initiation of treatment.

In the non-activated studies, the cells were exposed for 6, 20 or 44 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. In the 6 hour exposure group the treatment medium was removed after exposure, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with complete medium and returned to the incubator for an additional 14 hours. Two hours prior to the scheduled cell harvest at 20 or 44 hours after the initiation of treatment, Colcemid® was added to the duplicate flasks for each treatment condition at a final concentration of 0.1 µg/ml and the flasks returned to the incubator until cell collection.

In the S9 activated studies, the cells were exposed for 6 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator for an additional 14 or 38 hours. Two hours prior to the scheduled cell harvest, at 20 or 44 hours after the initiation of treatment, Colcemid® was

added to the duplicate flasks for each treatment condition at a final concentration of 0.1 µg/ml. The flasks were then returned to the incubator until cell collection.

An evaluation of cell growth inhibition was performed for both the non-activated and S9-activated assays. After completion of test article treatment (and after the recovery period, for the 6 hour non-activated and S9-activated assays) the cells were harvested by trypsinization and counted using a Coulter Counter to determine the number of cells per unit volume. The cell viability of each flask was assessed using Trypan blue dye exclusion. The number of cells per unit volume and the cell viability were then used to calculate the percent of cell growth inhibition relative to the solvent control.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the non-activated and S9-activated studies by trypsinization. Cells were collected approximately 20 and 44 hours after initiation of treatment. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for approximately 4 to 8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored in fixative at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant fluid decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) were also recorded. Chromatid and

isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. In the delayed harvests, the percentage of polyploid and endoreduplicated cells was recorded per 100 metaphase cells. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted.

Evaluation of Test Results

The toxic effects of treatment are based upon cell growth inhibition relative to the solvent-treated control and are presented for the initial and independent repeat assays. The number and types of aberrations found are presented for each treatment group. The percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined was calculated for each group. The frequency of structural aberrations per cell (mean aberrations per cell) was also calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. The percentage of polyploid and endoreduplicated cells (numerical aberrations) per 100 metaphase cells was evaluated for the delayed harvest of the independent repeat assay.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations are increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A reproducible and significant increase at a single dose level will be considered positive. Test articles not demonstrating a statistically significant increase in aberrations will be considered negative.

Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Record and Specimen Archives

All raw data, draft and final reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc., Rockville, Maryland.

Solubility Test

DMSO was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at 500 mg/ml, the maximum concentration tested.

Initial Chromosome Aberration Assay

CHO cells were first exposed to the following concentrations of test article: 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/ml in both the absence and presence of an Aroclor-induced S9-activation system. The test article was soluble in treatment medium at all concentrations tested. The pH and osmolality of the highest concentration in treatment medium, 5000 µg/ml, were approximately 7.5 and 369 mmol/kg, respectively. The osmolality of the solvent (DMSO) in treatment medium was 402 mmol/kg.

The activity of Furfural in the induction of chromosome aberrations in CHO cells was tested using a 6 hour exposure in the absence of an exogenous source of metabolic activation, followed by a 14 hour recovery period. Toxicity of at least 50% cell growth inhibition was observed at dose levels 1500 µg/ml and higher (Table 1). The findings of the cytogenetic analysis are presented by treatment flask in Table 2 and summarized by group in Table 5. At the highest test concentration evaluated microscopically for chromosome aberrations, 1500 µg/ml, the mitotic index was 35% reduced relative to the solvent control. Dose level 5000 µg/ml was not evaluated due to excessive cell growth inhibition. Dose levels 1.5, 5, and 15 µg/ml were tested but not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose levels 150, 500, and 1500 µg/ml ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$). The percentage of aberrant cells in the MMC group was 13% ($p \leq 0.01$, Fisher's exact test).

The activity of Furfural in the induction of chromosome aberrations in CHO cells was tested using a 6 hour exposure in the presence of an exogenous source of metabolic activation followed by a 14 hour recovery period. Toxicity of at least 50% cell growth inhibition was observed at dose level 5000 µg/ml (Table 3). The findings of the cytogenetic analysis are presented by treatment flask in Table 4 and summarized by group in Table 5. At the highest test concentration evaluated microscopically for chromosome aberrations, 1500 µg/ml, the mitotic index was 82% reduced relative to the solvent control. Dose level 5000 µg/ml was not evaluated due to a lack of scorable cells. Dose levels 1.5, 5, and 15 µg/ml were tested but not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose levels 500 and 1500 µg/ml ($p \leq 0.05$ and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$). The percentage of aberrant cells in the CP group was 26% ($p \leq 0.01$, Fisher's exact test).

Independent Repeat Assay

Based on the results of the initial assay, dose levels of 94, 188, 375, 750, 1500, and 3000 µg/ml were selected for further study in the non-activated and S9-activated portions of the independent repeat assay. The test article exposure times were 20 and 44 hours in the non-activated test system and 6 hours in the S9-activated test system. In the non-activated 20 and 44 hour exposure groups the cells were harvested immediately after the completion of treatment. In the 6 hour S9-activated exposure groups the cells were harvested 20 and 44 hours after the initiation of treatment. The test article was soluble in treatment medium at all dose levels tested. The pH and osmolality of the highest concentration in treatment medium, 3000 µg/ml, were approximately 7.5 and 360 mmol/kg, respectively. The osmolality of the solvent (DMSO) in treatment medium was 412 mmol/kg. Toxicity (cell growth inhibition of at least 50%) was observed at dose levels 750 µg/ml and higher in the non-activated 20 exposure group and at dose levels 188 µg/ml and higher in the non-activated 44 hour exposure group (Tables 6 and 8). In the S9-activated test system, toxicity (cell growth inhibition of at least 50%) was observed at dose level 3000 µg/ml in the 20 hour cell harvest and at dose levels 375 µg/ml and higher in the 44 hour cell harvest (Tables 10 and 12).

The findings of the cytogenetic analysis for the non-activated study are presented by treatment flask in Tables 7 and 9 and summarized by group in Table 14. At the highest test concentrations evaluated for chromosome aberrations in the 20 and 44 hour treatment groups, 750 µg/ml and 375 µg/ml, the mitotic indices were 24% and 90% reduced relative to the solvent control, respectively. In the non-activated 20 hour exposure group, dose level 1500 µg/ml was not evaluated due to a lack of scorable cells and dose level 3000 µg/ml was not evaluated due to extreme toxicity. In the non-activated 44 hour exposure group, dose level 750 µg/ml was not evaluated due to a lack of scorable cells and dose levels 1500 and 3000 µg/ml were not evaluated due to extreme toxicity. This represents a deviation from the protocol as only three dose levels were evaluated for chromosome aberrations in the non-activated 44 hour exposure group. However, the statistically significant results obtained in both the non-activated 20 and 44 hour exposure groups of the independent repeat assay confirm the statistically significant results seen in the initial assay and this deviation was determined to not have had any significant effect on the study. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control, at dose levels 375 and 750 µg/ml in the non-activated 20 hour exposure group and at dose levels 188 and 375 µg/ml in the non-activated 44 hour exposure group ($p \leq 0.01$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$) in both the non-activated 20 and 44 hour exposure groups. The percentage of cells with numerical aberrations were significantly increased above that of the solvent control at dose levels 94 and 188 µg/ml in the 44 hour harvest ($p \leq 0.05$ and $p \leq 0.01$, respectively, Fisher's exact test). The percentage of aberrant cells in the MMC groups were 12% and 27.5% ($p \leq 0.01$, Fisher's exact test) for the 20 and 44 hour treatment groups, respectively.

The findings of the cytogenetic analysis for the S9-activated study are presented by treatment flask in Tables 11 and 13 and summarized by group in Table 14. At the highest test concentrations evaluated for chromosome aberrations in the 20 and 44 hour harvests,

750 µg/ml, the mitotic index was 86% and 90% reduced, respectively, relative to the solvent control. In the S9-activated 20 hour harvest dose levels 1500 and 3000 µg/ml were not evaluated due to lack of scorable cells and in the S9-activated 44 hour harvest groups, dose level 1500 µg/ml was not evaluated due to lack of scorable cells and 3000 µg/ml was not evaluated due to extreme toxicity. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose levels 94, 188, 375, and 750 µg/ml in the S9-activated 20 hour harvest group ($p \leq 0.01$, Fisher's exact test). The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose levels 188, 375, and 750 µg/ml in the S9-activated 44 hour harvest group ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$) in both the S9-activated 20 and 44 hour harvest groups. The percentage of cells with numerical aberrations were significantly increased above that of the solvent control at the 44 hour harvest at dose levels 94, 375 and 750 µg/ml ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.01$, respectively, Fisher's exact test). The Cochran-Armitage test was also positive for a dose-responsive trend ($p \leq 0.05$). The percentage of aberrant cells in the CP group were 68% and 85.5% ($p \leq 0.01$, Fisher's exact test) for the 20 and 44 hour harvests, respectively.

CONCLUSION

The positive, solvent and untreated controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, Furfural was concluded to be positive in the *in vitro* mammalian cytogenetics test.

REFERENCES

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Scott, D., N.D. Danford, B.J. Dean and D.J. Kirkland. 1990. Metaphase Chromosome Aberration Assays In Vitro. In: Basic Mutagenicity Tests: UKEMS Recommended Procedures. D.J Kirkland (ed). Cambridge University Press, New York, NY.

TABLE 1

CONCURRENT TOXICITY TEST USING FURFURAL IN
THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 6 HOUR TREATMENT, 20 HOUR HARVEST

Treatment[1] (µg/ml)	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	2.08	96%			
	B	2.02	95%	1.96	N/C	N/C
DMSO	A	2.54	100%			
	B	2.23	97%	2.35	100%	0%
Furfural 1.5 µg/ml	A	2.01	97%			
	B	1.92	95%	1.89	80%	20%
5 ug/mnl	A	2.09	95%			
	B	2.19	90%	1.98	84%	16%
15 µg/ml	A	2.14	96%			
	B	2.19	97%	2.09	89%	11%
50 µg/ml	A	2.31	96%			
	B	2.11	98%	2.14	91%	9%
150 µg/ml	A	2.01	94%			
	B	1.79	95%	1.79	76%	24%
500 µg/ml	A	1.15	97%			
	B	1.57	99%	1.34	57%	43%
1500 µg/ml	A	1.01	97%			
	B	1.05	97%	1.00	42%	58%
5000 µg/ml	A	0.44	34%			
	B	0.49	31%	0.15	6%	94%
MMC 0.08 µg/ml	A	1.71	96%			
	B	2.21	97%	1.89	97%	3%
MMC 0.15 µg/ml	A	2.21	98%			
	B	1.63	96%	1.86	95%	5%

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1oC.
[2]Viability determined by trypan blue dye exclusion.
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4]Growth index = (mean cells per flask treated group/mean cells per flask solvent control group or untreated control group for the positive control comparison), expressed as a percentage.
[5]Cell growth inhibition = 100% - % cell growth index.

TABLE 2
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH FURFURAL IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 6 HOUR TREATMENT, 20 HOUR HARVEST

Treatment[1,8]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations							Average Aberrations Per Cell[3,7]
					Gaps	Chromatid-type[4] Breaks	Chromatid-type[4] Exch	Chromosome-type[5] Breaks	Chromosome-type[5] Dic	Chromosome-type[5] Ring	Severely Damaged Cells[6]	
Untreated cells	A	7.4	100	1	1	0	0	0	1	0	0	0.010
	B	9.4	100	2	0	2	0	0	0	0	0	0.020
DMSO	A	7.4	100	0	0	0	0	0	0	0	0	0.000
	B	8.6	100	0	0	0	0	0	0	0	0	0.000
Furfural 50 µg/ml	A	7.4	100	0	0	0	0	0	0	0	0	0.000
	B	6.6	100	1	0	0	0	0	1	0	0	0.010
150 µg/ml	A	9.8	100	2	0	3	0	0	0	0	0	0.030
	B	9.4	100	3	0	3	0	0	0	0	0	0.030
500 µg/ml	A	8.8	100	4	0	2	0	0	2	0	0	0.040
	B	9.0	100	5	1	2	0	0	2	1	0	0.050
1500 µg/ml	A	5.6	100	8	1	7	2	0	0	0	0	0.090
	B	4.8	100	6	1	4	3	3	0	0	0	0.100
MMC 0.08 µg/ml	A	8.0	100	11	3	11	3	4	0	0	1	0.280
	B	6.4	100	15	0	10	7	2	0	0	0	0.190

[1]CHO cells were treated for 6 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.
[8]Dose levels of 1.5, 5, and 15 µg/ml were also used in the treatment system but were not required for microscopic analysis. Dose level 5000 µg/ml was not analyzed due to excessive toxicity.

TABLE 3

CONCURRENT TOXICITY TEST USING FURFURAL IN
THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 6 HOUR TREATMENT, 20 HOUR HARVEST

Treatment[1] (µg/ml)	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	1.28	100%			
	B	1.47	99%	1.37	N/C	N/C
DMSO	A	1.32	97%			
	B	1.35	96%	1.29	100%	0%
Furfural 1.5 µg/ml	A	1.32	96%			
	B	1.60	94%	1.38	107%	-7%
5 ug/ml	A	1.20	97%			
	B	1.24	98%	1.19	92%	8%
15 µg/ml	A	1.23	94%			
	B	1.45	95%	1.27	98%	2%
50 µg/ml	A	1.49	97%			
	B	1.47	96%	1.43	111%	-11%
150 µg/ml	A	1.35	92%			
	B	1.46	94%	1.30	101%	-1%
500 µg/ml	A	0.93	94%			
	B	0.98	99%	0.93	72%	28%
1500 µg/ml	A	1.02	97%			
	B	0.79	95%	0.87	68%	32%
5000 µg/ml	A	0.60	77%			
	B	0.68	81%	0.51	39%	61%
CP 10 µg/ml	A	0.87	90%			
	B	1.10	93%	0.90	66%	34%
CP 20 µg/ml	A	0.96	98%			
	B	0.79	95%	0.85	62%	38%

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 6 hours at 37±1oC.
[2]Viability determined by trypan blue dye exclusion.
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4]Growth index = (mean cells per flask treated group/mean cells per flask solvent control group or untreated control group for the positive control comparison), expressed as a percentage.
[5]Cell growth inhibition = 100% - % cell growth index.

TABLE 4
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH FURFURAL IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 6 HOUR TREATMENT, 20 HOUR HARVEST

Treatment[1,3]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[5]	Average Aberrations Per Cell[3,7]
					Chromatid-type[4]			Chromosome-type[5]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	9.2	100	0	1	0	0	0	0	0	0	0.000
	B	7.4	100	0	1	0	0	0	0	0	0	0.000
DMSO	A	14.6	100	2	0	1	0	3	0	0	0	0.040
	B	10.6	100	0	0	0	0	0	0	0	0	0.000
Furfural 50 µg/ml	A	9.4	100	5	1	3	0	1	1	1	0	0.060
	B	6.6	100	3	0	2	0	1	0	0	0	0.030
150 µg/ml	A	9.4	100	2	0	2	0	1	0	0	0	0.030
	B	10.4	100	0	0	0	0	0	0	0	0	0.000
500 µg/ml	A	4.0	100	6	2	3	0	4	0	1	0	0.080
	B	4.2	100	5	0	9	1	0	0	0	0	0.100
1500 µg/ml	A	4.0	100	11	5	26	0	3	0	0	0	0.290
	B	0.6	100	14	3	15	1	1	0	0	3	0.470
CP 25 µg/ml	A	1.0	100	23	0	26	5	12	0	0	2	0.630
	B	0.8	100	29	3	38	4	13	0	0	4	0.950

[1]CHO cells were treated for 6 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.
[8]Dose levels of 1.5, 5, and 15 µg/ml were also used in the treatment system but were not required for microscopic analysis. Dose level 5000 µg/ml was not analyzed due to a lack of scorable cells.

TABLE 5
SUMMARY

INITIAL ASSAY

Treatment (µg/ml)	S9 Activation	Treatment/ Harvest Time	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)
Untreated	-	6/20	8.4	200	0.015 ± 0.122	1.5
DMSO	-	6/20	8.0	200	0.000 ± 0.000	0.0
Furfural						
50 µg/ml	-	6/20	7.0	200	0.005 ± 0.071	0.5
150 µg/ml	-	6/20	9.6	200	0.030 ± 0.198	2.5*
500 µg/ml	-	6/20	8.9	200	0.045 ± 0.208	4.5**
1500 µg/ml	-	6/20	5.2	200	0.095 ± 0.396	7.0**
MMC 0.08 µg/ml	-	6/20	7.2	200	0.235 ± 0.913	13.0**
Untreated	+	6/20	8.3	200	0.000 ± 0.000	0.0
DMSO	+	6/20	12.6	200	0.020 ± 0.223	1.0
Furfural						
50 µg/ml	+	6/20	8.0	200	0.045 ± 0.231	4.0
150 µg/ml	+	6/20	9.9	200	0.015 ± 0.158	1.0
500 µg/ml	+	6/20	4.1	200	0.090 ± 0.513	5.5*
1500 µg/ml	+	6/20	2.3	200	0.380 ± 1.427	12.5**
CP 10 µg/ml	+	6/20	0.9	200	0.790 ± 1.938	26.0**

[1]Severely damaged cells were counted as 10 aberrations.
[2]*, $p \leq 0.05$; **, $p \leq 0.01$; Fisher's exact test.

TABLE 6
CONCURRENT TOXICITY TEST USING FURFURAL IN
THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 20 HOUR CONTINUOUS TREATMENT

Treatment[1] (µg/ml)	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	1.35	95%			
	B	1.50	91%	1.33	N/C	N/C
DMSO	A	1.53	91%			
	B	1.55	95%	1.43	100%	0%
Furfural 94 µg/ml	A	1.10	96%			
	B	1.18	95%	1.09	76%	24%
188 µg/ml	A	0.98	97%			
	B	0.88	97%	0.90	63%	37%
375 µg/ml	A	0.79	97%			
	B	0.80	95%	0.76	53%	47%
750 µg/ml	A	0.59	99%			
	B	0.72	99%	0.65	46%	54%
1500 µg/ml	A	0.66	91%			
	B	0.64	94%	0.60	42%	58%
3000 µg/ml	A	0.67	19%			
	B	0.64	19%	0.12	9%	91%
MMC, 0.08 µg/ml	A	1.04	90%			
	B	1.07	90%	0.95	72%	28%

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1oC.
[2]Viability determined by trypan blue dye exclusion.
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4]Growth index = (mean cells per flask treated group/mean cells per flask solvent control group or untreated control group for the positive control comparison), expressed as a percentage.
[5]Cell growth inhibition = 100% - % cell growth index.

TABLE 7
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH FURFURAL IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 20 HOUR CONTINUOUS TREATMENT

Treatment[1,8]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[5]	Average Aberrations Per Cell[3,7]
					Chromatid-type[4]			Chromosome-type[5]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	10.6	100	1	0	0	0	1	0	0	0	0.010
	B	6.8	100	1	0	1	0	0	0	0	0	0.010
DMSO	A	7.0	100	3	0	1	0	2	0	0	0	0.030
	B	7.0	100	0	1	0	0	0	0	0	0	0.000
Furfural 94 µg/ml	A	5.0	100	2	2	1	0	0	1	0	0	0.020
	B	5.6	100	4	1	3	1	0	0	0	0	0.040
188 µg/ml	A	5.4	100	2	2	2	0	0	0	0	0	0.020
	B	4.4	100	2	1	3	0	0	0	0	0	0.030
375 µg/ml	A	3.8	100	5	0	5	0	1	0	0	0	0.060
	B	4.6	100	8	2	6	0	3	0	1	1	0.200
750 µg/ml	A	6.6	41	17	4	12	0	0	0	0	0	0.293
	B	4.0	34	9	4	5	0	0	0	0	0	0.147
MMC 0.08 µg/ml	A	3.2	100	13	5	10	4	5	0	0	0	0.190
	B	5.0	100	11	3	9	2	2	0	0	0	0.130

[1]CHO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.
[8]Dose level 1500 µg/ml was not analyzed due to a lack of scorable cells and dose level 3000 µg/ml was not analyzed due to excessive toxicity.

TABLE 8
CONCURRENT TOXICITY TEST USING FURFURAL IN
THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 44 HOUR CONTINUOUS TREATMENT

Treatment[1] (µg/ml)	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	2.63	98%			
	B	2.81	96%	2.64	N/C	N/C
DMSO	A	2.39	94%			
	B	2.39	98%	2.29	100%	0%
Furfural 94 µg/ml	A	1.33	98%			
	B	1.33	95%	1.28	56%	44%
188 µg/ml	A	0.80	97%			
	B	0.88	93%	0.79	35%	65%
375 µg/ml	A	0.47	97%			
	B	0.46	95%	0.45	19%	81%
750 µg/ml	A	0.45	93%			
	B	0.48	93%	0.43	19%	81%
1500 µg/ml	A	0.31	13%			
	B	0.37	9%	0.04	2%	98%
3000 µg/ml	A	0.41	0%			
	B	0.39	0%	0.00	0%	100%
MMC, 0.08 µg/ml	A	1.06	99%			
	B	1.91	97%	1.45	55%	45%

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 44 hours at 37±1oC.
[2]Viability determined by trypan blue dye exclusion.
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4]Growth index = (mean cells per flask treated group/mean cells per flask solvent control group or untreated control group for the positive control comparison), expressed as a percentage.
[5]Cell growth inhibition = 100% - % cell growth index.

TABLE 9
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH FURFURAL IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 44 HOUR CONTINUOUS TREATMENT

Treatment[1,3] (μg/ml)	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[5]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	9.0	100	3	0	2	0	0	0	0	0	0	0.000
	B	9.4	100	4	0	2	0	0	0	0	0	0	0.000
DMSO	A	7.6	100	4	0	0	0	0	0	0	0	0	0.000
	B	8.4	100	3	0	2	0	0	0	0	0	0	0.000
Furfural 94 μg/ml	A	4.4	100	8	0	0	0	0	0	0	0	0	0.000
	B	5.6	100	11	1	2	0	0	1	0	0	0	0.010
188 μg/ml	A	4.4	100	14	4	4	7	1	0	0	0	0	0.080
	B	2.6	100	9	6	5	6	3	2	0	0	0	0.110
375 μg/ml	A	1.0	67	39	22	9	24	0	1	0	0	2	0.672
	B	0.6	84	19	19	6	26	3	1	0	0	1	0.476
MMC 0.08 μg/ml	A	6.2	100	3	26	4	23	15	5	0	1	2	0.640
	B	7.2	100	7	29	4	29	17	4	0	0	1	0.600

[1]CHO cells were treated for 44 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.
[8]Dose level of 750 μg/ml was not analyzed due to a lack of scorable cells and dose levels of 1500 and 3000 μg/ml were not scored due to extreme toxicity.
[9]Numerical aberrations for flask A scored out of 100 cells and for flask B scored out of 87 cells.

TABLE 10
CONCURRENT TOXICITY TEST USING FURFURAL IN
THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 6 HOUR TREATMENT, 20 HOUR HARVEST

Treatment[1] (µg/ml)	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	0.95	90%			
	B	0.98	91%	0.87	N/C	N/C
DMSO	A	0.98	97%			
	B	1.00	92%	0.93	100%	0%
Furfural 94 µg/ml	A	1.02	96%			
	B	0.94	94%	0.93	100%	0%
188 µg/ml	A	0.85	95%			
	B	0.83	91%	0.78	83%	17%
375 µg/ml	A	0.73	93%			
	B	0.71	94%	0.67	72%	28%
750 µg/ml	A	0.54	96%			
	B	0.56	94%	0.53	56%	44%
1500 µg/ml	A	0.54	91%			
	B	0.57	98%	0.53	56%	44%
3000 µg/ml	A	0.43	84%			
	B	0.43	90%	0.37	40%	60%
CP, 5 µg/ml	A	0.73	90%			
	B	0.72	89%	0.65	74%	26%

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 6 hours at 37±1oC.
[2]Viability determined by trypan blue dye exclusion.
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4]Growth index = (mean cells per flask treated group/mean cells per flask solvent control group or untreated control group for the positive control comparison), expressed as a percentage.
[5]Cell growth inhibition = 100% - % cell growth index.

TABLE 11
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH FURFURAL IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 6 HOUR TREATMENT, 20 HOUR HARVEST

Treatment[1,8]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[5]	Average Aberrations Per Cell[3,7]
					Gaps	Chromatid-type[4] Breaks	Exch	Chromosome-type[5] Breaks	Dic	Ring		
Untreated cells	A	4.8	100	2	2	0	1	3	0	0	0	0.040
	B	3.0	100	3	0	3	0	0	1	0	0	0.040
DMSO	A	3.2	100	2	1	1	0	0	1	0	0	0.020
	B	5.2	100	2	2	0	1	0	1	0	0	0.020
Furfural 94 µg/ml	A	2.6	100	9	1	5	0	2	3	0	0	0.100
	B	2.0	100	9	2	7	1	1	1	0	0	0.100
188 µg/ml	A	2.8	100	9	2	4	6	0	0	0	0	0.100
	B	2.2	100	7	0	4	5	0	1	0	0	0.100
375 µg/ml	A	0.4	100	9	1	6	4	0	2	1	0	0.130
	B	1.0	100	45	18	50	13	4	1	1	9	1.590
750 µg/ml	A	1.0	100	45	6	48	15	3	1	0	13	1.970
	B	0.2	12	83	0	8	1	0	0	0	5	4.917
CP 5 µg/ml	A	0.2	55	56	1	36	19	0	1	0	2	1.382
	B	0.6	45	36	2	17	7	3	0	0	1	0.822

[1]CHO cells were treated for 6 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.
[8]Dose levels of 1500 and 3000 µg/ml were not analyzed due to a lack of scorable cells.

TABLE 12
CONCURRENT TOXICITY TEST USING FURFURAL IN
THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 6 HOUR TREATMENT, 44 HOUR HARVEST

Treatment[1] (µg/ml)	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	2.24	93%			
	B	2.22	92%	2.06	N/C	N/C
DMSO	A	2.15	89%			
	B	2.28	92%	2.01	100%	0%
Furfural 94 µg/ml	A	1.95	91%			
	B	2.21	93%	1.91	95%	5%
188 µg/ml	A	2.20	93%			
	B	1.88	95%	1.91	95%	5%
375 µg/ml	A	0.78	86%			
	B	0.84	91%	0.72	36%	64%
750 µg/ml	A	0.48	71%			
	B	0.48	86%	0.38	19%	81%
1500 µg/ml	A	0.42	85%			
	B	0.47	83%	0.38	19%	81%
3000 µg/ml	A	0.44	68%			
	B	0.32	64%	0.25	13%	87%
CP, 5 µg/ml	A	0.99	92%			
	B	1.01	94%	0.93	45%	55%

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 6 hours at 37±1oC.
[2]Viability determined by trypan blue dye exclusion.
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4]Growth index = (mean cells per flask treated group/mean cells per flask solvent control group or untreated control group for the positive control comparison), expressed as a percentage.
[5]Cell growth inhibition = 100% - % cell growth index.

TABLE 13
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH FURFURAL IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 6 HOUR TREATMENT, 44 HOUR HARVEST

Treatment[1,3] (μg/ml)	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[5]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	7.4	100	0	1	0	1	0	0	0	0	0	0.010
	B	6.2	100	2	1	0	0	0	1	0	0	0	0.010
DMSO	A	3.0	100	0	0	1	0	0	0	0	0	0	0.000
	B	5.0	100	1	1	0	1	0	0	0	0	0	0.010
Furfural 94 μg/ml	A	2.6	100	3	2	2	0	0	0	2	0	0	0.020
	B	5.8	100	4	1	1	0	0	1	0	0	0	0.010
188 μg/ml	A	3.8	100	1	4	0	2	0	0	1	1	0	0.040
	B	4.2	100	1	3	0	1	0	6	0	0	0	0.070
375 μg/ml	A	3.8	100	33	43	4	35	7	25	3	2	8	1.520
	B	4.2	100	13	28	1	19	5	8	5	1	4	0.780
750 μg/ml	A	0.0	3	0	33	0	0	0	0	1	0	0	0.333
	B	0.8	100	16	49	4	34	33	9	1	0	16	2.370
CP 5 μg/ml	A	5.0	100	2	82	3	45	23	47	8	3	36	4.860
	B	4.2	100	3	89	1	43	25	43	5	5	45	5.710

[1]CHO cells were treated for 6 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.
[8]Dose level of 1500 μg/ml was not analyzed due to a lack of scorable cells and dose level of 3000 μg/ml was not analyzed due to extreme toxicity.

TABLE 14
SUMMARY

INDEPENDENT REPEAT ASSAY

Treatment (µg/ml)	S9 Activation	Treatment/ Harvest Time	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations (%) Numerical[2]	Cells With Aberrations (%) Structural
Untreated	-	20/20	8.7	200	0.010 ± 0.100		1.0
DMSO	-	20/20	7.0	200	0.015 ± 0.122		1.5
Furfural							
94 µg/ml	-	20/20	5.3	200	0.030 ± 0.171		3.0
188 µg/ml	-	20/20	4.9	200	0.025 ± 0.186		2.0
375 µg/ml	-	20/20	4.2	200	0.130 ± 0.798		6.5**
750 µg/ml	-	20/20	5.3	75	0.227 ± 0.649		13.3**
MMC 0.08 µg/ml	-	20/20	4.1	200	0.160 ± 0.475		12.0**
Untreated	-	44/44	9.2	200	0.000 ± 0.000	3.5	0.0
DMSO	-	44/44	8.0	200	0.000 ± 0.000	3.5	0.0
Furfural							
94 µg/ml	-	44/44	5.0	200	0.005 ± 0.071	9.5*	0.5
188 µg/ml	-	44/44	3.5	200	0.095 ± 0.527	11.5*	5.0**
375 µg/ml	-	44/44	0.8	151	0.563 ± 1.676	2.1[4]	20.5**
MMC 0.08 µg/ml	-	44/44	6.7	200	0.620 ± 1.482	5.0	27.5**
Untreated	+	6/20	3.9	200	0.040 ± 0.281		2.5
DMSO	+	6/20	4.2	200	0.020 ± 0.140		2.0
Furfural							
94 µg/ml	+	6/20	2.3	200	0.100 ± 0.332		9.0**
188 µg/ml	+	6/20	2.5	200	0.100 ± 0.375		8.0**
375 µg/ml	+	6/20	0.7	200	0.860 ± 2.187		27.0**
750 µg/ml	+	6/20	0.6	112	2.286 ± 3.610		49.1**
CP 5 µg/ml	+	6/20	0.4	100	1.130 ± 1.931		68.0**
Untreated	+	6/44	6.8	200	0.010 ± 0.100	1.0	1.0
DMSO	+	6/44	4.0	200	0.005 ± 0.071	0.5	0.5
Furfural							
94 µg/ml	+	6/44	4.2	200	0.015 ± 0.122	3.5*	1.5
188 µg/ml	+	6/44	4.0	200	0.055 ± 0.391	1.0	3.5*
375 µg/ml	+	6/44	4.0	200	1.195 ± 2.563	23.0**	35.5**
750 µg/ml	+	6/44	0.4	103	2.311 ± 3.540	15.5**	48.5**
CP 5 µg/ml	+	6/44	4.6	200	5.285 ± 4.148	2.5	85.5**

[1]Severely damaged cells were counted as 10 aberrations.
[2]*, p≤0.05; **, p≤0.01; Fisher's exact test.
[3]Data not collected for 20 hour harvest time.
[4]Percentage calculated out of 187 total cells.

APPENDIX I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES
STRUCTURAL ABERRATIONS
1993-1995

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.1%	1.2%	34.9%
Standard Deviation	1.0%	1.2%	21.8%
Range	0.0% to 5.5%	0.0% to 6.0%	8% to 100.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.6%	1.5%	45.4%
Standard Deviation	1.3%	1.3%	24.8%
Range	0.0% to 5.5%	0.0% to 6.0%	7.5% to 100.0%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, PBS, 2% DMSO in saline, CSEP Buffer, citrate buffer, culture medium, 1% human albumin, extraction blanks, human platelets, placebo, 5% dextrose, 0.75% NaCMC, bicarbonate buffered 0.9% saline, sham electrodes.

[2]Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 μg/ml,) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 μg/ml), and Mitomycin C (MMC, 0.08-0.15 μg/ml).

[3]Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 μg/ml), and benzo(α)pyrene, (B[α]P, 30 μg/ml).

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS

1993-1995
HISTORICAL CONTROL VALUES
TOTAL NUMERICAL ABERRATIONS

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.5%	1.7%	2.8%
Standard Deviation	1.4%	1.4%	2.8%
Range	0.0% to 6.0%	0.0% to 5.5%	0.0% to 15.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.6%	1.8%	3.8%
Standard Deviation	1.3%	1.5%	3.6%
Range	0.0% to 6.5%	0.0% to 7.5%	0.0% to 16.5%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, PBS, CSEP Buffer, 1% human albumin, human platelets, 5% dextrose, 0.75% NaCMC, bicarbonate buffered 0.9% saline.
[2]Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 μg/ml), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 μg/ml), and Mitomycin C (MMC, 0.08-0.15 μg/ml).
[3]Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 μg/ml), and benzo(α)pyrene (B[α]P, 30 μg/ml).

APPENDIX II

Study Protocol

QA APPROVED

PROTOCOL AMENDMENT 1

SPONSOR: [ ]

TEST ARTICLE I.D.: Furfural

MA STUDY NO: [ ] (Protocol No.: SPGT335)

PROTOCOL TITLE: *In Vitro* Mammalian Cytogenetic Test With an Independent Repeat Assay

---

1. LOCATION: Page 2 of 9, § 4.3; Study Director:

   AMENDMENT: Amend the name of the Study Director to Ramadevi Gudi, Ph.D.

   REASON FOR THE AMENDMENT: To reflect a change of Study Director.

   EFFECTIVE DATE: June 1, 1996

2. LOCATION: Page 2 of 9, § 7.0; Experimental Design and Methodology:

   AMENDMENT: Amend the first sentence to read "...CHO cells will be exposed to eight concentrations of test article...".

   REASON FOR THE AMENDMENT: To correlate sections 7.0 and 7.2.

APPROVALS:

Elizabeth A Smalley for EG
STUDY DIRECTOR

6/14/96
DATE

[ ]

6/24/96
DATE

MICROBIOLOGICAL ASSOCIATES, INC.

PROTOCOL AMENDMENT II

QA APPROVED 9-20-96

SPONSOR: [ ]

TEST ARTICLE I.D.: Furfural

MA STUDY NO: [ ](Protocol No.: SPGT335)

PROTOCOL TITLE: *In Vitro* Mammalian Cytogenetic Test With an Independent Repeat Assay

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1. **LOCATION:** Page 5 of 9, § 7.5.3; Positive Controls:

   AMENDMENT: Amend the cyclophosphamide test concentration range to 5-25 μg/ml.

   REASON FOR THE AMENDMENT: In order to avoid the extreme toxicity seen using the higher dose levels of CP with a 6 hour exposure.

APPROVALS:

STUDY DIRECTOR	9/20/96 DATE
[ ]	9/20/96 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

pem 5-9-96 QA APPROVED

Received by RA/QA 5-6-96

MA Study Number: [ ]

In Vitro Mammalian Cytogenetic Test With an Independent Repeat Assay

1.0 PURPOSE

The purpose of this study is to test the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: [ ]

2.2 Address: [ ]

2.3 Representative: [ ]

2.4 Sponsor Project #:

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **Furfural**

3.2 Controls:

Negative:	Untreated Cells
Solvent:	Solvent Vehicle
Positive:	Mitomycin C (MMC), Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

3.4 Test Article Retention Sample.

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Patrick T. Curry, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 6-18-96

5.2 Proposed Experimental Completion Date: 9-6-96

5.3 Proposed Report Date: 9-20-96

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Evans, 1976).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

In the initial chromosome aberration assay, CHO cells will be exposed to six concentrations of the test article as well as positive and negative controls in duplicate cultures for 6 hours in the absence and presence of an S-9 reaction mixture. The concentrations tested will be selected by evaluating cytotoxicity after test article treatment in a dose range finding assay. The dividing cells will be harvested at a single time point, approximately 1.5 times the normal cell cycle time, i.e., 20 hours from the initiation of treatment. The four highest dose levels that are acceptable will be evaluated microscopically for structural chromosome aberrations.

In the independent repeat chromosome aberration assay, CHO cells will be exposed to six concentrations of the test article as well as positive and negative controls in duplicate cultures for approximately 20 and 44 hours in the absence of an exogenous source of metabolic activation and for 6 hours in the presence of an S-9 reaction mixture. The concentrations tested will be selected based on the results of the initial assay. The dividing cells, arrested in metaphase, will be harvested for microscopic evaluation of chromosome aberrations approximately 20 and 44 hours after the initiation of treatment. The four highest dose levels that are acceptable will be evaluated microscopically for structural chromosome aberrations. The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group (Preston et al., 1981).

The experimental design is summarized as follows:

Assay	Treatment Condition	Treatment Time	Harvest Time
Initial Assay	Non-activated	6 Hr	20 Hr
	S-9 Activated	6 Hr	20 Hr
Independent repeat Assay	Non-activated	20 Hr	20 Hr
		44 Hr	44 Hr
	S-9 Activated	6 Hr	20 Hr
		6 Hr	44 Hr

7.1 Solubility determination

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to determine the maximum soluble concentration up to 500 mg/ml. Vehicles compatible with this test system, in order of preference, include but are not limited to sterile, distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to 500 mg/ml.

7.2 Dose Selection

Selection of the dose levels for the cytogenetics assay will be based upon cell growth inhibition after treatment in an initial chromosome aberration assay. CHO cells will be exposed to solvent alone and to at least 8 concentrations of test article. The highest concentration tested will be 5 mg/ml for freely soluble test articles (solubility will be determined by visual inspection without the aid of magnification), or the maximum concentration resulting in a workable suspension for poorly soluble test articles. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for approximately 6 hours in the absence and presence of S-9. The cells will be harvested 20 hours after treatment initiation. The cells will be harvested by trypsinization and counted using a Coulter counter and cell viability will be assessed using trypan blue dye exclusion. The cell and viability counts will be used to determine cell growth inhibition relative to the solvent control. Just prior to trypsinization the cell cultures will be visually inspected for the extent of monolayer confluency relative to the solvent control.

Whenever possible, the highest dose level used for evaluation in the initial cytogenetic assay will be selected to give at least 50% toxicity (cell growth inhibition). For precipitating and toxic test articles, doses will be selected on the basis of toxicity and will include at least one non-precipitating dose level and at least one non-toxic dose level. For precipitating non-toxic test articles, doses will be selected to include a precipitating dose level only at the highest dose evaluated. Precipitating dose levels will be selected such that the precipitate does not interfere with evaluation. At least three additional dose levels will be included in the initial (and independent repeat) assay. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, if excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if osmolality of the treatment medium is excessive, the Sponsor will be consulted.

7.3 Frequency and Route of Administration

Target cells will be treated for approximately 6, or 20 and 44 hours in the non-activated studies and for 6 hours in the S9-activated studies. Treatment will be achieved by incorporation of the test article-solvent mixture into the medium. This technique has been demonstrated to be an effective method of detection of chemical clastogens in *in vitro* test systems (Galloway et al., 1994).

7.4 Metabolic Activation System

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The S-9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$), 6 mM potassium chloride (KCl), 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S-9 per ml serum free medium.

7.5 Controls

7.5.1 Untreated Control

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. For solvents other than water or saline, the final concentration in treatment medium will not exceed 1%.

7.5.3 Positive Controls

Mitomycin C will be used as the concurrent positive control in the non-activated study for the initial and independent repeat cytogenetic assays. Cyclophosphamide will be used as the concurrent positive control in the S-9 activated study for the initial and independent repeat cytogenetic assays. In the chromosome aberration assays, two concentrations of either MMC (within the range of 0.08 to 0.15 µg/ml) or CP (within the range of 10 to 50 µg/ml) will be used.

7.6 Preparation of Target Cells

Exponentially growing CHO cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 ug streptomycin/ml) for each treatment condition at approximately 5×10^5 cells/25 cm^2 flask for cell collection times of 24 hours or less and at 3×10^5 cells/25 cm^2 flask for collection times in excess of 24 hours. The flasks will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the test system will be identified by the study number, the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated exposure or 5 ml S-9 reaction mixture for the activated exposure, to which will be added 50 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water or medium is used as the solvent. An untreated control consisting of cells in complete medium or S-9 reaction mixture will be also included.

7.8.1 Initial Assay

In the non-activated and S-9 activated studies, the cells will be exposed for 6 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the exposure period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator for an additional 14 hours. Cells will be collected at 20 hours after initiation of the 6 hour treatment. An aliquot of the cell suspension will be collected and counted using a Coulter counter and trypan blue dye exclusion. The cell and viability counts will be used as a concurrent toxicity test to determine cell growth inhibition relative tc the solvent control.

7.8.2 Independent Repeat Assay

In the non-activated study, the cells will be exposed for approximately 20 and 44 hours. The cells will be exposed at 37 ± 1° C in a humidified atmosphere of 5 ± 1% CO_2 in air. Cells will be collected at 20 and 44 hours after initiation of treatment.

In the S-9 activated study, the cells will be exposed for 6 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the exposure period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator for an additional 14 or 38 hours. Cells will be collected at 20 and 44 hours after initiation of the test article treatment.

In both the non-activated and S-9 activated studies, an aliquot of the cell suspension will be collected and counted using a Coulter counter and trypan blue dye exclusion. The cell and viability counts will be used as a concurrent toxicity test to determine cell growth inhibition relative to the solvent control.

7.9 Collection of Metaphase Cells

Cells will be collected by trypsinization approximately 20 hours after initiation of treatment in the initial chromosome aberration assay and approximately 20 and 44 hours after initiation of treatment in the independent repeat chromosome aberration assay. Harvest times have been selected to reflect one and a half complete cell cycles following treatment (20 hours) and a delayed harvest 24 hours later (44 hours). Two hours prior to harvest, Colcemid will be added to the cultures at a final concentration of 0.1 µg/ml. Cells will be collected by centrifugation, swollen with 0.075 M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-6°C. To prepare slides, the cells will be collected by centrifugation, resuspended in fresh fixative. The fixed cells will be dropped onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. One to two slides will be prepared from each treatment flask and allowed to air dry. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

Slides will be scanned for scorable metaphases. The highest dose level with an acceptable toxicity level and solubility profile, and at least three lower dose levels, will be evaluated microscopically for structural chromosome aberrations. A single concentration of the positive control will be analyzed.

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20±2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) will be examined from negative and positive control cultures; and vehicle and test article treated cultures, and will be scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations will be recorded using a calibrated microscope stage. In the delayed harvest, the number of polyploid and endoreduplicated cells will be evaluated per 100 cells. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The frequency of cells with structural chromosome aberrations in the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water is used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon cell growth inhibition relative to the solvent-treated control and will be presented for both the initial and the independent repeat assay. The number and types of structural chromosome aberrations found, the percentage of cells with structural chromosome aberrations (percent aberrant cells) in the total population of cells examined, the overall structural chromosome aberration frequency and the mean structural chromosome aberrations per cell will be calculated and reported for each treatment group. The number of polyploid and endoreduplicated cells will also be reported and the total percentage of cells with numerical aberrations will be calculated. Chromatid and isochromatid gaps will be presented in the data but not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells will be performed using the Fisher's exact test. Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage trend test will be used to measure dose-responsiveness.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response if the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A reproducible and significant increase at a single dose level also will be considered positive. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates Inc. and will accurately describe all methods used for generation and analysis of the data. Results presented will include, but not be limited to:

- cells used
- test conditions: composition of medium, CO_2 concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive and negative controls
- number of cell cultures
- number of metaphases analyzed (method for determination; data given separately for each culture)
- cell growth inhibition and mitotic index
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with the U.S. FDA Redbook II (1993); EU Legislation L 383 a/148, B.10 Mutagenicity (*In Vitro* Mammalian Cytogenetic Test), December, 1992; OECD Guideline 473 (Genetic Toxicology: In Vitro Mammalian Cytogenetic Test), May, 1983; Japanese MHW guidelines (Notification No. 24 of the Pharmaceutical Affairs Bureau, Japanese Ministry of Health and Welfare (JMHW),

1989; and EPA Health Effects Testing Guidelines. Subpart 798.5375 (In Vitro Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies.

Will this study be submitted to a regulatory agency? yes
If so, to which agency or agencies? EU, TSCA, AND JAPAN

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Evans, H.J. 1976. Cytological methods for detecting chemical mutagens. In: Chemical Mutagens, Principles and Methods for their Detection, vol. 4. A. Hollaender (ed). Plenum Press, New York, NY.

Galloway, S.M., M.J. Aardema, M. Ishidate, J.L. Ivett, D.J. Kirkland, T. Morita, P. Mosesso and T. Sofuni. 1994. Report from working group on *in vitro* tests for chromosomal aberrations. Mutation Res. 312:241-261.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff, J.S. Wassom. 1981. Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program. Mutation Res. 87:143-188.

Scott, D., N.D. Danford, B.J. Dean and D.J. Kirkland. 1990. Metaphase Chromosome Aberration Assays *In Vitro*. In: Basic Mutagenicity Tests: UKEMS Recommended Procedures. D.J Kirkland (ed). Cambridge University Press, New York, NY.

14.0 APPROVAL

[ ] 16 April 1996
DATE

Patrick T. Cimino
MA STUDY DIRECTOR

5-6-96
DATE